Antimitogenic and chemosensitizing effects of the methyltransferase inhibitor zebularine in ovarian cancer

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Abstract

Deoxycytosine methylation within CpG islands of tumor suppressor genes plays a prominent role in the development and progression of drug-resistant ovarian cancer. Consequently, epigenetic therapies directed toward tumor suppressor demethylation/reexpression could potentially reverse malignant phenotypes and chemosensitize recalcitrant tumors. In this report, we examined the demethylating agent zebularine [1-(3-o-ribofuranosyl)-1,2-dihydropyrimidin-2-one], in comparison with the well-known methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC), for its ability to inhibit ovarian cancer cell proliferation and to demethylate and induce tumor suppressor genes. Zebularine exerted significant (>5-aza-dC) antiproliferative effects against the ovarian cancer cell lines Hey, A2780, and the cisplatin-resistant A2780/CP in a dose-dependent manner (65% versus 35% inhibition at 48 hours, zebularine versus 5-aza-dC). Moreover, 48-hour treatment with 0.2 mmol/L zebularine significantly induced demethylation of the tumor suppressors ras-associated domain family 1A and human MutL homologue-1. RASSF1A gene reexpression was also observed, as was reexpression of two other tumor suppressors, ARHI and BLU, although levels differed from those induced by 5-aza-dC. Global analyses of DNA methylation revealed similar overall demethylation (2.5- to 3-fold) by 5-aza-dC and zebularine as determined by methyl acceptance assay. However, differences in demethylation of individual loci were observed as determined by differential methylation hybridization. Finally, we found that zebularine could re sensitize the drug-resistant cell line A2780/CP to cisplatin, with a 16-fold reduction in the IC50 of that conventional agent. In summary, zebularine seems to be a promising clinical candidate, singly or combined with conventional regimens, for the therapy of drug-resistant ovarian cancer.

Introduction

The acquisition of drug resistance is a severe impediment to the successful therapy of ovarian cancer. Although most patients initially respond to a taxane/platinum regimen, relapse occurs in 80% of cases following a median period of 15 months (1). Although chemoresistance has a number of causative factors, one frequent determinant is the positive selection of clones harboring genotypes that allow survival from the intended drug insult (2, 3). Such genotypes can be inherent to the tumor or acquired during treatment but generally involve up-regulation of antiapoptotic oncogenes and/or down-regulation of proapoptotic tumor suppressors (3). Tumor suppressors are often rendered nonfunctional by deletion, mutation, or translocation; however, in a growing number of cases, loss of function is associated with nongenetic (i.e., epigenetic) modifications (4), primarily DNA methylation.

Aberrations of DNA methylation are now accepted as a common instigator in the development and progression of many cancers (4), including ovarian cancer (5–7). In general, neoplasia is correlated with overall genomic hypomethylation, whereas isolated, normally unmethylated CG-rich regions, known as “CpG islands” (often associated with transcriptionally active genes), frequently become hypermethylated (8). Tumor suppressor genes such as ras-associated domain family 1A (RASSF1A), p16, and human MutL homologue-1 (hMLH1) are frequently silenced by CpG island methylation in numerous tumor types (9); the subsequent interruption of proapoptotic pathways is thought to contribute to increased proliferation and/or drug resistance (2). In particular, loss of mismatch repair gene expression, including that of hMLH1, is believed to result in inadequate response to platinum agents, resulting in enhanced resistance to those therapies (10). As both hMLH1 and RASSF1A are believed to promote apoptotic responses (9, 11), their silencing likely contributes significantly to the progression of drug-resistant ovarian cancer.

To reverse methylation-induced silencing of tumor suppressors, a number of inhibitors are currently under investigation in clinical and preclinical trials (12). In several
studies to date, demethylation-induced reactivation of a number of tumor suppressors has been shown both in vitro and in vivo (8, 13). In one study, the well-known methylation inhibitor 5-aza-2’-deoxycytidine (5-aza-dC) induced hMLH1 reexpression and reversal of drug resistance in mouse xenografts of the cisplatin-resistant ovarian cancer cell line A2780/CP (10). In clinical trials, 5-aza-dC has shown effectiveness against acute promyelocytic leukemia and myelodysplastic syndrome (14). Potential disadvantages, however, of 5-aza-dC, are its disappointing results against solid tumors (15), its relative instability (resulting in lack of p.o. availability; ref. 16), and its potential deamination (and thus inactivation) by the enzyme cytidine deaminase (17).

One inhibitor of cytidine deaminase is the cytidine analogue zebularine [1-((D-ribofuranosyl)-1,2-dihydropyrroimidin-2-one; ref. 18]. Zebularine is similar in structure to azacytidine except that it lacks a 4’ amino group and a 5’ nitrogen (Fig. 1A). Besides inhibiting cytidine deaminase, zebularine was also found to form covalent complexes with DNA methyltransferases (DNMT; similar to 5-aza-dC; ref. 19). Zebularine was further shown, by p.o. administration, to reactivate a silenced p16 gene and elicit in vivo tumor suppression of human T24 bladder cancer cells in mouse xenografts (20). A follow-up study, also in T24 cells, found that zebularine could cause significant demethylation of CpG-rich regions and, following 5-aza-dC treatment, could effectively inhibit remethylation (21).

In this study, we examined the effects of zebularine on a number of ovarian carcinoma cell lines, including Hey, A2780, and A2780/CP. Zebularine showed significant antiproliferative activity against those cancer lines and minimal toxicity to normal ovarian epithelial cells. Additionally, zebularine pretreatment enhanced cisplatin sensitivity in A2780/CP cells, substantially reducing the IC50 of that chemotherapeutic drug. Although zebularine treatment led to promoter demethylation of several tumor suppressors, demethylation did not always lead to gene reexpression. Moreover, although zebularine and 5-aza-dC elicited similar levels of global DNA demethylation, we found, using a microarray approach, differences in specific loci. In summary, this in vitro study strongly supports the potential of zebularine as a viable clinical candidate.

Materials and Methods
Reagents
Zebularine (NSC309132) was supplied by Dr. Victor Marquez (Developmental Therapeutic Program, National Cancer Institute, Frederick, MD). 5-Aza-dC, sodium metabisulfite, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and hydroquinone were purchased from Sigma (St. Louis, MO). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA) or HyClone (Logan, UT). Primers (sequences in Supplementary Data) were obtained from MWG Biotech (High Point, NC) and quantitative PCR reagents were from Roche (Indianapolis, IN). Molecular biology reagents were purchased from Qiagen (Valencia, CA), Promega (Madison, WI), or New England BioLabs (Beverly, MA).

Cell Culture and Proliferation Assays
A2780 and Hey cells were purchased from American Type Culture Collection (Manassas, VA), whereas cisplatin-resistant A2780/CP cells were a kind gift from Dr. Robert Brown (Cancer Research UK, Beatson Laboratories, Glasgow, United Kingdom). A2780, A2780/CP, and Hey cells were maintained as we have described previously (22). Normal ovarian surface epithelial cells were obtained from patients by gently scraping off the ovarian surface epithelium and expanding the cells in short-term culture (two to four passages; ref. 23). To measure cell proliferation, cells were seeded in 96-well plates at a density of 104 per well and treated the following day with zebularine or 5-aza-dC as described in Results. Following drug treatment, cells were exposed to MTT (Sigma; ref. 24) for 4 hours, solubilized in DMSO, and the MTT metabolite formazan was quantitated at 600 nm using a Bio-Tek (Winooski, VT) ELX-800 microplate absorbance reader. For cisplatin treatment, cells were pretreated with zebularine for 48 hours, treated with various doses of cisplatin for 3 hours, and allowed to recover for 72 hours before MTT quantitation.

Gene Expression Studies
Following treatment of A2780/CP cells for 48 hours with 5 μmol/L 5-aza-dC or 0.2 mmol/L zebularine, RNA was isolated using RNeasy mini kits (Qiagen). Reverse transcription was then carried out and, to determine relative gene expression, quantitative PCR was done essentially as we have described previously (25, 26), using primers (sequences available in supplement) specific for each gene of interest with cDNA template and LightCycler quantitation.

Cell Cycle Analysis
Cells were trypsinized, pelleted, and fixed in cold 70% ethanol. For fluorescent labeling, a solution of propidium iodide and RNase in PBS was used. Flow cytometric analysis was done on a Becton Dickinson FACSCalibur (Becton Dickinson, San Diego, CA). Propidium iodide fluorescence was measured using doublet discrimination by collecting and plotting the FL2-width and FL2-area signals. ModFit, by Verity software (Topsham, ME), was used to quantitate the percentage of cells in G0-G1, S, and G2-M phases of the cell cycle.

DNA Bisulfite Treatment and Methylation-Specific PCR
For methylation analyses, DNA was purified from untreated or drug-treated cells using DNeasy mini kits (Qiagen) and quantitated by absorbance at 260 nm. To deaminate unmethylated cytosines, and thus distinguish methylated from unmethylated alleles, 10 μg genomic DNA were incubated for 16 hours at 50°C with 2.5 mol/L sodium bisulfite in the presence of 1 mol/L hydroquinone.
Following overnight treatment, impurities were removed using a Wizard Clean-up system (Promega). Methylation-specific PCR was then done, as we have described previously (25, 28). Briefly, 40 ng bisulfite-treated DNA were amplified separately by primer pairs specific to methylated or unmethylated alleles of each gene, with products then electrophoresed on 2.0% agarose, stained, and photographed. Methylation-specific PCR primer sequences are available in Supplementary Data.

**Methyl Acceptance Assay**

Genomic DNA was isolated from A2780/CP cells treated with vehicle, 5 μmol/L 5-aza-dC, and 0.2 and 0.5 mmol/L zebularine. Methyl acceptance was done according to the method of Balaghi and Wagner (29). Briefly, 0.5 μg DNA was incubated with 3 μmol/L [14C]-adenosylmethionine (2 μCi) and 3 units CpG methylase in 10 mmol/L Tris-HCl (pH 7.9), 120 mmol/L NaCl, 10 mmol/L EDTA, and 1 mmol/L DTT. The reaction was carried out at 30°C for 1 hour followed by entrapment on Whatman DE81 filters and scintillation counting (29). 14C-DNA incorporation into DNA from drug-treated cells was normalized to incorporation into DNA from untreated cells and depicted as a fold increase over control.

**Differential Methylation Hybridization**

Differential methylation hybridization was done essentially as we have described previously (28, 30), with the exception that the restriction enzyme McrBC (31) was used in place of HpaII. Briefly, 2 μg genomic DNA (from vehicle- or drug-treated A2780/CP cells) were digested with MseI, possessing a restriction site of A^TAA and located outside of most CpG islands. 5' Overhangs were then filled in to produce blunt ends, followed by ligation to the linkers 5'-GCGGTGACCCGGGAGATCTGAATTC-3' and 5'-GAATTCAGATC-3'. DNA was then digested (or mock-digested) with McrBC, having a recognition site of 5'-(G/A)3,000(G/A)-3' and digesting only methylated DNA. This step effectively eliminates (by restriction) methylated template DNA before PCR amplification using 24 cycles of 95°C × 30 seconds, 55°C × 30 seconds, and 72°C × 60 seconds. Mock- and McrBC-digested samples were then incubated with aminooallyl-dUTP, dATP, dCTP, dGTP, and Klenow fragment, followed by labeling with Cy3 (McrBC-digested DNA) or Cy5 (mock digest). Cy3- and Cy5-labeled amplicons were then combined and cohybridized to a 12,192-loci (12K) CGI microarray (Dr. Sandy Der, University of Toronto, Toronto, Ontario, Canada) overnight at 65°C, washed, and scanned. Microarray analysis was done using GenePix Pro 4.0 (Axon Instruments, Union City, CA).

**Differential Methylation Hybridization Microarray Analysis**

To analyze microarray data (three separate images), the circular features mode of GenePix was used for each feature on the 12K array. Spots representing repetitive clones were then “flagged” and unacceptable features were removed by filtering. Nonrepeated clones were then normalized to a subset of mitochondrial genes, as those would be expected to be completely unmethylated. These normalization factors were then used to standardize methylation (Cy5/Cy3) ratios, and loci with ratios >1.7 were accepted as significantly methylated.

**Results**

**Growth Inhibition by Zebularine**

In this study, we examined the antiproliferative effects of zebularine (structure in Fig. 1A), a nucleoside analogue and cytidine deaminase inhibitor, against several ovarian carcinoma cell lines. To this end, Hey, A2780, and the cisplatin-resistant A2780/CP ovarian cancer cells were
treated for 48 hours with increasing doses of zebularine. Growth inhibition was determined by MTT assay (24). As shown in Fig. 1B, zebularine elicited >65% growth inhibition at 200 μmol/L zebularine treatment in all three cell lines. IC_{50} values were as follows: A2780/CP, 152 μmol/L; A2780, 91 μmol/L; and Hey, 64.7 μmol/L. These effective zebularine doses were similar (for A2780/CP) or somewhat lower (for A2780 and Hey) than those observed for T24 bladder cancer cells (32). In A2780/CP cells, zebularine was also compared with the well-established demethylating agent 5-aza-dC (structure in Fig. 1A). In contrast to zebularine, 5-aza-dC, at nontoxic doses, achieved only 35% growth inhibition of A2780/CP cells (Fig. 1C).

As a previous study of 5-aza-dC showed cell cycle arrest at low doses, and apoptosis at higher doses (33), we did flow cytometry analysis of A2780/CP cells treated with zebularine. As shown in Fig. 2, a low dose (0.1 mmol/L) caused a slight increase in the proportion of cells in G2 phase, from 16.5% to 23.3%, whereas a higher dose (0.5 mmol/L) treatment resulted in likely apoptosis, as measured by an increase in cells with sub-2N material, from 4.8% to 21%. To further indicate zebularine-induced apoptosis, we found increased caspase activity (an early apoptotic event), after a 12-hour treatment, at doses below 200 μmol/L (data not shown).

In previous studies, zebularine was found less toxic to fibroblasts than to various cancer cells (20, 34). To determine the general cytotoxicity of zebularine to nonmalignant ovarian cells, we assessed its effect on normal ovarian surface epithelial cells. As these cells divide more slowly than ovarian cancer cell lines, we treated normal ovarian surface epithelial cells with increasing doses of zebularine for 96 hours rather than for 48 hours. As shown in Fig. 1B (gray unfilled columns), even after 96 hours, normal ovarian surface epithelial growth rates were reduced by only 35% at 200 μmol/L zebularine, in contrast to 65% inhibition of the three cancer cell lines after 48 hours of treatment. This value (35%) is similar to that found previously for general cytotoxicity to T24 bladder cancer cells (20). In normal ovarian surface epithelial cells, zebularine exhibited an IC_{50} of 417 μmol/L following 96-hour treatment, 2.7- to 6.4-fold higher than the 50% effective doses for the 48-hour-treated cancer lines.

**Tumor Suppressor Demethylation and Reexpression**

It is now believed that the antiproliferative effects of DNA methylation inhibitors are largely due to the reexpression of tumor suppressor genes (35). Previously, zebularine was shown, by microarray, to up-regulate a number of apoptosis-related genes in multiple myeloma cells (36). Here, we examined zebularine induction of several likely methylated tumor suppressor genes; this induction was compared with up-regulation elicited by 5-aza-dC. First, the tumor suppressor gene hMLH1, encoding a DNA mismatch repair enzyme, implicated in

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**Figure 2.** Flow cytometry analysis of untreated A2780/CP cells, with analysis for distribution between G0-G1, G2-M, and S phases; debris indicates cells with sub-2N nuclear material. **A,** untreated A2780/CP cells; **B,** A2780/CP cells treated with 0.1 mmol/L zebularine for 48 h; **C,** A2780/CP cells treated with 0.5 mmol/L zebularine for 48 h; and **D,** histogram summarizing alterations in cell cycle (summary of A–C).
cisplatin-induced apoptosis (37), was examined. This gene has been firmly established as methylated and silenced in numerous cancers (9), and induced by 5-aza-dC in ovarian cancer cell lines and xenografts (10). In agreement with those previous findings, we found that hMLH1 was induced >250-fold by 5-aza-dC in A2780/CP cells; however, we did not observe significant reexpression mediated by zebularine (Fig. 3A). By contrast, the tumor suppressor RASSF1A, also firmly established as methylated in numerous cancer types (11), was induced 3.6-fold by zebularine but not by 5-aza-dC (Fig. 3B). Although others have shown 5-aza-dC reexpression of RASSF1A, in renal cell carcinoma cells (38), that induction occurred after 5 days of treatment (our treatment was only for 48 hours). The imprinted tumor suppressor gene ARHI (7) was up-regulated 3.6-fold by 5-aza-dC and 2.5-fold by zebularine (Fig. 3C). On the other hand, BLU, a tumor suppressor gene of unknown function (39), was induced 6.2-fold by zebularine but not significantly by 5-aza-dC (Fig. 3D).

To examine demethylation by zebularine, compared with 5-aza-dC, we used methylation-specific PCR to analyze the methylation status of CpG islands within the promoter regions of RASSF1A and hMLH1. As shown in Fig. 3E, both zebularine and 5-aza-dC elicited detectable demethylation of both genes following 48-hour treatment. As methylation-specific PCR is primarily a qualitative methylation assay, it remains possible that there were quantitative demethylation differences engendered by these two agents. Thus, hMLH1, although demethylated by zebularine, was not significantly induced (Fig. 3A), demonstrating that, for this gene, promoter demethylation alone is not sufficient for reexpression.

**Effects on Global and Localized Genomic Methylation**

To investigate a possible basis for the differential gene induction effects of zebularine and 5-aza-dC, we examined changes in global methylcytosine levels elicited by each drug. For this purpose, we used a technique known as methyl acceptance assay in which [14C]-adenosylmethionine acts as a methyl donor for bacterial CG methylase-catalyzed DNA methylation of genomic DNA. Incorporation of labeled methyl groups is inversely correlated with the methylation status of the DNA before treatment (i.e., highly methylated DNA is unable to accept additional methyl groups). As shown in Fig. 4, 5 μmol/L 5-aza-dC and 0.2 and 0.5 mmol/L zebularine increased 14C-methyl incorporation by 2.5- to 3-fold over untreated cells (P < 0.05). No significant differences between the various dose/drug treatments were observed.

To examine demethylation in more detail at the single gene level, we performed a technique previously developed by our group, known as differential methylation hybridization (28, 30). This technique involves cohybridization of Cy5-labeled (mock-digested, reference) and Cy3-labeled (digested, test) amplified amplicons to a microarray of CpG islands. Methylated amplicons are digested by the methylation-specific endonuclease McrBC, resulting in exclusive binding of the Cy5 (mock-digested) reference-labeled amplicons (40). Correspondingly, the ratio of Cy5/

Figure 3. Evaluation of reexpression and demethylation of tumor suppressor genes by 5-aza-dC or zebularine. For gene reexpression, quantitative PCR analysis was done on coding regions of hMLH1 (A), RASSF1A (B), ARHI (C), and BLU (D). Columns, mean of three replicates; bars, SE. *, significant (P < 0.05) difference from untreated cells. E, methylation-specific PCR was done within promoter regions of hMLH1 and RASSF1A using bisulfite-treated DNA from untreated, 5-aza-dC, or zebularine-treated A2780/CP cells. U, unmethylated amplicons; M, methylated amplicons.
demethylation of a common set of 78 single-copy loci, zebularine exclusively demethylated eight loci and 32 were solely demethylated by 5-aza-dC. These results suggest mechanistic differences in methylation inhibition by the two agents.

Zebularine-Induced Cisplatin Sensitization

In advanced-stage ovarian cancer, chemoresistance seems to be due, at least in part, to methylation-induced silencing of drug response genes. Consequently, demethylating agents have previously been examined for their ability to reverse such silencing and facilitate resensitization. To examine this potential effect using zebularine, we examined resensitization of cisplatin-resistant A2780/CP cells. Cells were pretreated with increasing doses of zebularine (0–200 μmol/L) for 48 hours, followed by 3-hour treatment with varying doses of cisplatin (0–80 μmol/L), 3 days recovery, and MTT assay. Zebularine elicited a dose-dependent decrease in cell survival at each cisplatin dose (Fig. 6A). Similarly, 5-aza-dC induced a dose-dependent sensitization to cisplatin (Fig. 6B) at likely physiologically achievable levels. Figure 6C and D depicts the resulting decreases in effective dose (IC50) of cisplatin as a function of concentration of zebularine and 5-aza-dC, respectively. As shown, zebularine treatment resulted in a maximal IC50 decrease to ~6 μmol/L (Fig. 6C), whereas 5-aza-dC achieved a reduction to ~20 μmol/L (Fig. 6D).

Discussion

Aberrations in DNA methylation are now well established in the process of tumorigenesis and the acquisition of drug resistance. The reversal of such methylation, to allow reexpression of silenced tumor suppressors, is one promising strategy for tumor regression and chemosensitization. A strong candidate now in several clinical trials is the nucleoside analogue 5-aza-dC, also known as decitabine or Dacogen (14). 5-aza-dC has shown impressive effects in vitro, eliciting antiproliferative effects on numerous tumor cell lines, including those of the bladder, lung, breast, colon, and pancreas (42). In whole animals, 5-aza-dC has shown efficacy against non–small-cell lung cancer and bile duct tumor models (42). 5-Aza-dC is believed to induce apoptosis through mitochondrial-dependent pathways (43) although the role of p53 remains controversial (43, 44). This drug was recently approved by the Food and Drug Administration for the treatment of myelodysplastic syndrome, for which it has shown impressive effectiveness (14).

Although 5-aza-dC holds much promise for hematologic malignancies, its effectiveness against human solid tumors has thus far been disappointing. In a 101-patient phase II study of various tumors, only one response was observed (45). Similar discouraging results were observed for testicular, hormone-independent prostate, and ovarian cancers (15). The basis for poor solid tumor activity is unclear but is likely due to decreased exposure (46) and lower growth fractions (47) compared with blood cancers (46).
Besides questions regarding solid tumor efficacy, other negative aspects of 5-aza-dC are myelosuppression (48) and neutralization by cytidine deaminase in drug-resistant tumors (17). Instability and acid lability (16) has prevented the development of a p.o. formulation of 5-aza-dC, necessitating administration by infusion. Although lower-dose 5-aza-dC treatments were as effective as, or more effective than, previously used higher doses, myelosuppression could not be evaluated for those regimens (14). Zebularine, by contrast, is much more stable (49) and its p.o. delivery was effective in mice (20); however, i.v. administration may be necessary in other species (50).

To compare growth inhibition by zebularine versus 5-aza-dC, we examined the effects of each agent on proliferation of the ovarian cancer cell lines Hey, A2780, and A2780/CP. Zebularine, at 200 μmol/L, elicited >65% growth inhibition of all three cancer lines but only 35% inhibition of normal ovarian epithelial cells (Fig. 1B), suggesting minimal toxicity. In a recent report, zebularine was found similarly nontoxic to normal fibroblasts but selectively toxic to T24 bladder cancer cells. In that study, it was shown that several normal (but not malignant) cells express only low levels of a uridine/cytidine kinase necessary for zebularine phosphorylation, which is required for DNA incorporation (34). It is possible that this is the case in normal versus malignant ovarian epithelial cells. In aggressive A2780/CP70 cells, our flow cytometry and caspase analyses suggested the occurrence of apoptosis at higher zebularine doses and a slight G2 arrest at lower doses (Fig. 2). Based on our proliferation assay, however, it is unlikely that zebularine induces substantial cell cycle arrest or apoptosis in normal ovarian epithelial cells (Fig. 1B).

To evaluate possible mechanisms of antigrowth activity, we examined demethylation and reexpression of tumor suppressor genes. Previously, zebularine was shown to elicit reexpression of one silenced tumor suppressor gene, p16, in T24 bladder cancer cells (20, 21). Although we did not observe zebularine induction of p16 in ovarian cancer cells (data not shown), in which methylation of that gene is controversial (51, 52), we detected reexpression of other tumor suppressor genes by quantitative PCR analysis, including three genes implicated in ovarian cancer progression—RASSF1A, BLU, and ARHI (7, 11, 39). RASSF1A and another tumor suppressor gene, hMLH1, were both shown by methylation-specific PCR as demethylated by zebularine treatment (Fig. 3E); that treatment, however, did not

Figure 6. Zebularine and 5-aza-dC resensitization of cisplatin-resistant A2780/CP cells. Growth curves for 0, 10, 20, 40, and 80 μmol/L cisplatin (cis) are shown for increasing (48 h) pretreatment dosages of zebularine (A) and 5-aza-dC (B). Points, mean of at least six replicates; bars, SE. To summarize the growth curves, the decrease in IC50 of cisplatin is shown as a function of zebularine (C) and 5-aza-dC dosages (D). Growth was determined by MTT assay. Points, at least six replicates; bars, 95% confidence levels.
not result in hMLH1 reexpression (Fig. 3A). It is possible that silenced hMLH1 transactivating factors are induced by 5-aza-dC but not by zebularine. In support of such a hypothesis, a recent study by our group found that 5-aza-dC treatment resulted in up-regulation of a number of methylated genes; however, several unmethylated genes were also found induced (53). Such an effect could be due to up-regulation of transcription factors that subsequently induce downstream target genes. Several examples of such methylated transcription factor genes exist, including HOXA5 (54) and members of the GATA family (55). Thus, it is possible that in our zebularine-treated cells, despite demethylation of its promoter, failure to induce essential hMLH1 transactivating factors results in continued gene silencing.

Although we did not observe hMLH1 reexpression as a result of zebularine-induced demethylation, dose-dependent zebularine pretreatment of cisplatin-resistant A2780/CP cells did result in increased drug sensitization (Fig. 6A and C). Thus, it seems that alternative cascades (besides mismatch repair) may allow for enhanced response to DNA-damaging agents. Indeed, response to platinum adducts is multifactorial, with both down-regulation of mismatch repair and up-regulation of nucleotide excision repair pathways (56). Consequently, it is possible that zebularine acts to impede nucleotide excision repair or restore p53 activity by unknown mechanisms unrelated to activity of hMLH1. In support of this conjecture, a recent study showed that zebularine increased radiosensitivity of tumor cells, likely by inhibition of DNA repair (57).

Although 5-aza-dC treatment resulted in sensitization to cisplatin in our system (similar to zebularine), clinical trials of that combination have been quite discouraging, resulting in significant toxicity (58, 59). It remains unclear whether a zebularine/cisplatin combination would be similarly toxic, although the apparent different mechanisms of the two demethylation agents allows for the possibility that zebularine would be less detrimental.

Similar to our hMLH1 methylation-specific PCR results, we also observed similar extents of demethylation by the two agents at the whole genome level (Fig. 4). However, by microarray, we observed 78 genes commonly demethylated by both agents, with 32 exclusive to 5-aza-dC and only eight specific for zebularine (Fig. 5C), in agreement with a previous study demonstrating only a small number of genes up-regulated by zebularine (34). Thus, it is quite possible that these two agents exert their demethylating (and antiproliferative) effects by quite different mechanisms. In one recent study, it was found that zebularine acted similarly to 5-aza-dC in irreversible binding to, and depletion of, DNMT-1 (21). In that same study, however, only transient (and much lesser) down-regulation of DNMT-3a and DNMT-3b was observed (21). Recently, it was reported that DNMT-3a/DNMT-3b double-null embryonic stem cells were more resistant to 5-aza-dC that DNMT-1 null cells, suggesting that those isoforms (DNMT-3a and DNMT-3b) preferentially mediate cellular responses to that demethylating agent (60). As studies to date have shown zebularine preference for DNMT-1 over DNMT-3a and DNMT-3b (21), it is possible that the divergent effects of zebularine, compared with 5-aza-dC, are due to differences in methyltransferase specificity.

It is also possible that the effects of zebularine are mediated by factors other than DNA demethylation. In a recent clinical trial of 5-aza-dC for hematopoietic malignancies, it was found that demethylation of the cell cycle regulator p15, in patient lymphocytes, had no correlation with survival (14). Another cell cycle regulator, p21, which possesses no promoter methylation, was found up-regulated by 5-aza-dC in a lung cancer cell line, likely mediating growth arrest in those cells (61). Additionally, as zebularine is a ribonucleoside (in contrast to 5-aza-dC, a deoxyribonucleoside), its incorporation into RNA has been recently shown as 7-fold higher than DNA incorporation (32). RNA alteration could result in altered transcription and/or ribosomal disruption, similar to 5-azacytidine (62). Although RNA incorporation (and disrupted transcription/translation) could result in significant toxicity to normal cells, we and others (20, 34) did not observe such detrimental effects. This limited toxicity could be due to differential RNA incorporation between normal and cancer cells, with cancer cells overexpressing enzymes such as uridine/cytidine kinase, which facilitate zebularine insertion into nucleic acids (32). In a study of another cytidine analogue, gemcitabine, RNA and DNA incorporation was found to vary widely among various cells lines (63); such differential incorporation would likely lead to highly divergent cellular responses.

In summary, zebularine is an impressive antiproliferative agent against ovarian cancer cell lines. The mechanisms responsible for these effects are likely complex and quite dissimilar from those of the well-known demethylating agent, 5-aza-dC. In combination with conventional chemotherapeutic drugs, such as platinum agents, zebularine may hold promise for the treatment of drug-resistant tumors; such combinations may allow for reduced, achievable in vivo dosages with significant clinical efficacy.

References
Molecular Cancer Therapeutics

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